CAMPYLOBACTER RECTUS VIRULENCE AND THE EFFECTS OF IMMUNIZATION IN THE PREGNANT MOUSE MODEL

by
Mehul H. Gadhia, DMD

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Approved by

Advisor: Professor Steven Offenbacher

Advisor: Dr. Deborah Threadgill

Advisor: Dr. David Paquette

ABSTRACT

MEHUL GADHIA: *Campylobacter rectus* Virulence And The Effects Of Virulence In The Pregnant Mouse Model

(Under the direction of Dr. Offenbacher)

Previous studies in the mouse model have reported that maternal infection with *Campylobacter rectus*, a periodontal pathogen, is associated with intrauterine fetal growth restriction. To that end, we hypothesized that maternal immunization prior to challenge with *C. rectus* would protect the fetus from growth restriction. Prior to mating, mice in one treatment arm received immunization via an intra-chamber challenge of heat-killed *C. rectus* 314. On gestation day (Gd) 7.5, pregnant mice in the treatment groups received an intra-chamber challenge of live *C. rectus* at concentrations of 10^9 CFU/ml. Pregnant mice were sacrificed on Gd 16.5 and fetuses evaluated for weight, and crown-rump length. Maternal serum was collected and maternal antibody response was evaluated. Fetuses from the *C. rectus* challenged group had shorter crown-rump lengths and weighed significantly less than the immunized group and the control group. Maternal serum of the immunized group displayed marked elevations of IgG antibody to *C. rectus* compared to the non-immunized and control group. In conclusion, immunization of pregnant dams with heat-killed *C. rectus* provides a robust maternal IgG response and protects against fetal growth restriction.

In addition, *C. rectus* virulence factors that may have a possible role in fetal growth restriction were investigated. The genes *peb4*, *cadF*, *cdtB* and *ciaB* that have been identified as virulence genes and sequenced in other *Campylobacter* species were examined for their

presence in *C. rectus*. To that end, the genome sequences of 4 *Campylobacter* spp. (*C. jejuni*, *C. lari*, *C. coli* and *C. upsaliensis*) were used to design degenerate oligonucleotide primers to attempt detection and isolation of these genes in *C. rectus*. Although a match was not found at the nucleotide level, investigation of matches at the protein level revealed similarities with cytochrome c552 of *C. jejuni* RM1221, NADP-dependent malic enzyme from *C. fetus* subsp. *fetus* and mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase from *C. fetus* subsp. *fetus*. This is significant for future attempts to obtain genomic sequences from *C. rectus* and warrants further study.

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LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
bp	Base pairs
cadF	Campylobacter adhesion to fibronectin gene
CDT	Cytolethal distending toxin
cdtB	Cytolethal distending toxin gene
CFU	Colony forming unit
ciaB	*Campylobacter* invasion antigen gene
crs	*Campylobacter rectus* S-layer gene
csx	*Campylobacter rectus* S-layer RTX gene
C. coli	*Campylobacter coli*
C. fetus	*Campylobacter fetus*
C. jejuni	*Campylobacter jejuni*
C. lari	*Campylobacter lari*
C. rectus	*Campylobacter rectus*
C. sputorum	*Campylobacter sputorum*
C. upsaliensis	*Campylobacter upsaliensis*
ddNTPs	dideoxynucleotides
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FGR	Fetal growth restriction
Fn	Fibronectin

Gd	Gestation day
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IUB	International union of biochemistry
kDa	Kilodalton
LBW	Low birth weight
OD	Optical density
P. gingivalis	*Porphyromonas gingivalis*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
peb1-4	*campylobacter* adhesion gene
PLBW	Preterm low birth weight
RTX	Repeats in the structural toxin
S-layer	Surface layer
SE	Standard error
SPB	Spontaneous preterm birth
TAE	Tris-acetate-EDTA
TBST	Tris-buffered saline tween-20
V. bubulus	*Vibrio bubulus*
V. fetus	*Vibrio fetus*

REVIEW OF THE LITERATURE

Adverse Pregnancy outcomes

Despite advances in prenatal care, the occurrence of adverse pregnancy outcomes, which include spontaneous preterm births (SPB) and associated low birth weight (LBW) infants has not decreased in decades [1, 2]. These advances include extensive use of tocolytic drugs such as α-adrenergic agents, magnesium sulfate, prostaglandin synthase inhibitors and calcium channel blockers administered to arrest premature contractions [3]. Even with intervention, SPB (defined as delivery at less than 37 weeks of gestation) accounts for about 10 percent of all live births and often results in LBW (defined as infants born less than 2,500 grams or about 5.5 lbs) which is a significant cause of infant mortality [4]. This subset of deliveries is referred to as premature low birth weight (PLBW). PLBW is an economic, as well as a societal burden. The annual cost of treating PLBW infants in intensive care units is more than five billion dollars. Moreover, PLBW has been associated with long term morbidity for the survivors including respiratory distress, cerebral palsy and learning disabilities [4]. Traditional risk factors for adverse pregnancy outcomes include poor overall maternal health, previous preterm birth, smoking, alcohol consumption, maternal emotional stress and infection [3].

Infection, inflammation and adverse pregnancy outcomes

Support for the role of infection in adverse pregnancy events comes from many sources including the fact that many biological markers for preterm labor are also the same biomarkers of infection [5-7]. Gibbs in 2001 [3] reviewed the possible association between adverse pregnancy outcomes and infection/inflammation and summarized the following: 1)histological chorioamnionitis is increased in preterm births; 2)clinical infection is increased after preterm birth; 3)there are significant associations of lower genital tract organisms and infections with preterm birth or preterm premature rupture of the membranes; 4)there are positive cultures of amniotic fluid or membranes from some patients with preterm labor and preterm birth; 5)there are markers of infections in preterm birth; 6)bacteria or their products induce preterm birth in animal models.

In humans, some infectious agents, including rubella, *Toxoplasma* and *Treponema*, have been shown to be able to cross the placental barrier, leading to the induction of fetal immunological responses to these infecting agents [8-10]. Compromise of the placental barrier is extremely serious since the developing fetus lacks a fully competent immune system to fend off infections.

Maternal-fetal transfer of immunoglobulins begins around the 17th week of gestation and gradually increases in proportion to gestational age. It is generally accepted that the only antibody to cross the placenta is Immunoglobulin G. IgM, IgA and IgE have not been shown to be transferred [11]. Additional support for the involvement of infectious agents in this pathology is provided in veterinary sciences where it is well established that transient infertilities, spontaneous abortions and premature births in animals are often caused by various bacteria and their products.

Periodontal disease and adverse pregnancy outcomes

Periodontal disease is often described as a complex bacterial infection that affects the supporting structures of the teeth. This ubiquitous disease has been defined as a "specific mixed infection which causes periodontal destruction in the appropriately susceptible host" [12]. Recent evidence suggests an association between periodontal disease and adverse pregnancy outcomes [13-15]. Epidemiological evidence suggests that pregnant women with periodontal disease are seven times more likely to have preterm labor than their periodontally healthy counterparts [16].

Studies with pregnant rodent models have demonstrated that low-grade challenges with oral organisms during pregnancy resulted in impaired fetal growth. This was demonstrated using a chronic subcutaneous infection model with *Porphyromonas gingivalis* or *Campylobacter rectus* and also in a model of experimental periodontitis [16-19]. Since there are no animal models of preterm birth, these data provide important proof-of-concept experiments that raise the possibility that distant, low grade oral infections might also trigger inflammation of the human maternal-fetal unit in a manner analogous to that seen with reproductive tract infections.

Molecular evidence for an association between periodontal disease and adverse pregnancy events comes from the analysis of maternal and fetal antibody response to periodontal pathogens. A strong maternal IgG response to the "Red" complex subgingival plaque organisms comprised of the pathogens *Porphyromonas gingivalis*, *Tannerela forsythensis* and *Treponema denticola* protects the fetus from exposure to these oral bacteria. A decreased maternal IgG response to "Red" cluster organisms is correlated with an increased exposure of the fetus to the "Orange" cluster of subgingival plaque organisms of which *C.*

rectus is a member. Exposure of the fetus to *C. rectus*, as indicated by a strong fetal IgM response to this bacterium, is associated with an elevated risk of adverse pregnancy outcomes [20].

Campylobacter Species

History and taxonomy

In 1963, Sebald and Véron [21] transferred *V. fetus* and *V. bubulus* into a new genus, *Campylobacter*, due to their low DNA base composition, their microaerophilic growth requirements, and their nonfermentative metabolism. This is considered the creation of the genus *Campylobacter*. Ten years later, Véron and Chatelain [22] reported four distinct species in the genus: *Campylobacter fetus* (the type species), *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter sputorum*. More names were introduced through the years and during the past decade, the taxonomy of *Campylobacter* has undergone drastic changes. The two main reasons for this phenomenon are the increased interest in *Campylobacter*-like organisms and the drastic evolution of bacterial taxonomy due to technological advances in molecular biology, biochemistry and affiliated disciplines. While previous classification systems were based mainly on biochemical and morphological criteria, present-day classifications are primarily phylogeny based since they are constructed around a backbone derived from similarity studies of highly conserved macromolecules such as rRNA genes [23]. These rRNA homology studies evaluated and revised bacterial classification schemes based on the degree of rRNA cistron similarity.

In 1987, Romaniuk et al. [24] and Lau et al. [25] presented the first phylogenetic data on *Campylobacter* species. They compared partial 16S rRNA sequences of *C. fetus*, *C. jejuni*, *C.*

coli, *C. lari*, *C. sputorum*, and *C. pylori* strains and found that these species formed a previously undescribed bacterial lineage, which related to other gram-negative bacteria only by very deep branching.

In 1991, Vandamme et al. [26] proposed a complete revision of the taxonomy and nomenclature of the genus *Campylobacter* and related bacteria after an extensive DNA-rRNA hybridization study. The genus was restricted to the rRNA homology cluster containing *C. fetus*, the type species of the *genus Campylobacter*. Among others, the generically misnamed *Wolinella recta* was included in the emended genus as *Campylobacter rectus*.

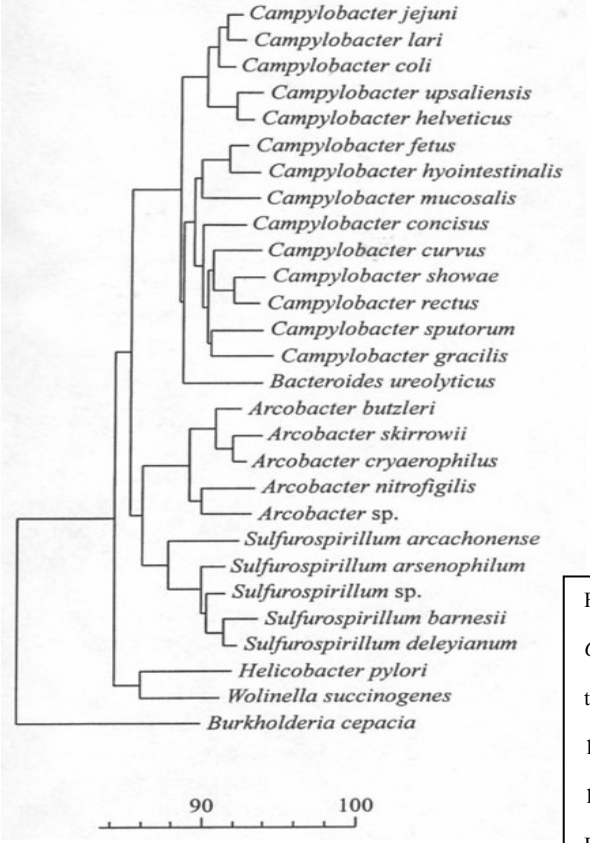

Figure 1. Phylogenetic tree of the family *Campylobacteraceae* and its closest phylogenetic neighbors, the genera *Helicobacter* and *Wolinella*, based on the percent 16S rRNA gene sequence similarity. The scale represents a 10% sequence similarity. (Adapted from: Nachamkin and Blaser, 2000. *Campylobacter*, 2nd Edition)

Campylobacter rectus Virulence

Campylobacter rectus, formerly *Wolinella recta* [26], is a putative, gram-negative, anaerobic, motile bacterium which is associated with several forms of human periodontal diseases [27-30]. Although there is a limited amount of information available concerning the molecular mechanisms and genetic characteristics of *C. rectus*, some genes responsible for its pathogencity have been identified. The surface layer (S-layer) and cytotoxic activity have been characterized and thought to be its major virulence factors. This S-layer protein is assumed to be involved in resistance of *C. rectus* to phagocytic uptake and to bactericidal activity of serum [31, 32]. Recently, the gene encoding the S-layer protein (*crs*) has been sequenced [32]. The cytotoxic activity associated with *C. rectus* was suggested to be due to a

pore-forming protein toxin belonging to the RTX (repeats in the structural toxin) family [33]. Two closely related genes, *csxA* and *csxB* (for *C. rectus* S-layer and RTX protein) have been cloned from *C. rectus* and characterized [34].

S-layer

The paracrystalline cell surface layer (S-layer) appears to be composed of a single protein [35, 36]. These S-layer proteins form regularly arranged structures on the outer surface of various bacteria. They are assumed to play a role in virulence of several pathogens by rendering the bacteria resistant to complement killing and providing structures for adherence to host cells [37]. Although the first 15 amino acids of the S-layer proteins are identical in several strains of *C. rectus*, the molecular mass of the S-layer protein varies from 150 to 166 kDa among strains [38-40]. By peptide analysis, Nitta et al. [38] have also reported that there is internal sequence heterogeneity between the S-layer proteins from strains 314 and ATCC 33238.

Evidence that the *C. rectus* S-layer is a virulence factor stems from studies of a strain of *C. rectus* that lost its S-layer during long-term in vitro subculture [40]. It was reported that the S-layer negative bacteria were more adherent to human gingival fibroblasts than were other strains of *C. rectus* with intact S-layers [40]. In addition, strains which had been passaged 15 to 17 times *in vitro* formed smaller lesions in a mouse abscess model for soft tissue destruction than did low-passage *C. rectus* strains [41]. These studies suggest that the *C. rectus* S-layer helps the organism evade host defense mechanisms.

csx

The cytotoxic activity of gram-negative bacteria such as that proposed for *C. rectus* [42] is often due to pore-forming protein toxins belonging to the family of RTX (repeats in the structural toxin) proteins. RTX toxins, which include the α-hemolysin (HlyA) of *Escherichia coli* [43], the leukotoxins (LktA) of *Pasteurella haemolytica* [44], AaltA of *Actinobacillus actinomycetemcomintans* [45], the cytotoxins ApxIA, ApxIIA, and ApxIIIA of *Actinobacillus pleuropneumoniae* [46], and the adenylate cyclase CyaA of *Bordetella pertussis* [47], are the major virulence factors associated with these pathogens [48, 49].

Campylobacter rectus was suggested to contain genes encoding RTX proteins as shown by Kuhnert et al.[42]. Furthermore, Gillespie et al. [33] described a cytotoxic fraction of *C. rectus* which reacted serologically with antiserum directed against leukotoxin of *A. actinomycetemcomitans*. Recent literature described the presence of two new genes in *C. rectus* coding for bistructural S-layer-RTX proteins named *csxA* and *csxB* (*C. rectus* S-layer-RTX protein) [34]. This study reported that these genes that code for csx proteins appear to be bi-functional and possess two structurally different domains. The N-terminal part shows similarity with S-layer protein, especially *crs* of *C. rectus*. The C-terminal part comprising most of *csxA* and *csxB* is a domain with 48 and 59 glycine-rich canonical nonapeptide repeats, respectively, arranged in three blocks. Purified recombinant csx peptides bind Ca^{2+}. These are characteristic traits of RTX toxin proteins. The S-layer and RTX domains of *csx* are separated by a proline-rich stretch of 48 amino acids. Interestingly, all *C. rectus* isolates studied in this report contained copies of either the *csxA* or *csxB* gene or both. Also, *csx* genes were absent from all other *Campylobacter* species examined. [34]

Campylobacter jejuni, *C. coli* and *C. fetus* Virulence

It is widely accepted that cell adherence, invasive capabilities and toxin production by *Campylobacter* is multifactorial and several potential virulence determinants have been proposed [50, 51].

Campylobacter adherence

In order for this pathogenic bacterium to evade the host's defensive mechanisms, establish an infection, and damage host cells, it must first adhere to the host tissue. Bacterial adherence is mediated by bacterial cell surface structures called adhesins, which recognize specific receptors on the particular host cell surface. A number of outer-membrane adhesion proteins have been identified. In *C. jejuni*, the best-characterized adhesins to date include *cadF* and *peb1* [52-56].

cadF

The target of the *cadF* adhesin is fibronectin (Fn), a component of the extracellular matrix [52]. Fn appears to be a common host cell target and numerous pathogens, including *C. jejuni* [52, 53, 54, 57], *Staphylococcus aureus* [58, 59], *Streptococcus pyogenes* [60, 61], *Salmonella enterica serovar Enteritidis* [62], *Escherichia coli* [63, 64], *Neisseria gonorrhoeae* [65], *Mycobacterium avium* [66], and *Treponema* species [67-69], possess Fn binding ability.

The binding of *Campylobacter jejuni* to fibronectin (Fn), is mediated by a 37 kDa outer-membrane protein termed cadF for *Campylobacter* adhesion to fibronectin [52]. The specificity of *C. jejuni* binding to Fn, via the *cadF* gene, was demonstrated using antibodies reactive against Fn and cadF [70]. Also, consistent with previous articles from this group of

authors, it has been reported that *cadF* gene and protein is conserved among *C. jejuni* and *C. coli* isolates [53, 54].

peb1-4

The purification of four proteins from glycine-extracted material of *C. jejuni* has been described [55]. These four proteins have molecular masses of 28, 29, 30, and 31 kDa and are named PEB1, PEB2, PEB3, and PEB4, respectively. The importance of these proteins in adherence and invasion is reported in the literature.

The PEB1 protein is encoded by the *peb1A* locus. A *C. jejuni peb1A* null mutant exhibited a 50- to 100-fold reduction in binding and a 15-fold reduction in internalization to HeLa cells compared to the *C. jejuni* parental isolate [56]. This study also reported that the mutant exhibited a reduction in the duration of mouse intestinal colonization compared to the parental isolate.

Campylobacter invasion

ciaB

Previous work has demonstrated that *C. jejuni* synthesizes a set of proteins during coculture with epithelial cells, a subset of which are secreted. The secreted proteins have been collectively referred to as *Campylobacter* invasion antigens (cia proteins) and the gene identified was named *ciaB* [54]. These proteins appear to be responsible for invasion of the organism into mammalian cells [71]. Specifically, insertional mutagenesis of the *ciaB* gene encoding a 73-kDa secreted protein (CiaB) results in a significant reduction in the number of *C. jejuni* cells internalized into mammalian cells compared to a *C. jejuni* wild-type isolate.

The absence of the ciaB protein secretion in the *C. jejuni ciaB* mutant is specific, as the invasive phenotype of this organism is restored by complementation in *trans* with the *ciaB* gene [71].

Campylobacter toxicity

cdtB

The best characterized of the toxins attributed to *Campylobacter* spp. is the cytolethal distending toxin (CDT). Johnson and Lior first reported CDT production by *Campylobacter* [72]. They assayed over 700 *Campylobacter* strains, including 583 *C. jejuni*, 109 *C. coli*, 16 *C. lari*, and 7 *C. fetus* strains for CDT activity. Approximately 40 % of each of these species was found to produce active CDT and no correlation was found between CDT production, serotype, biotype or country of origin of the tested strains. They also reported that CDT activity in culture supernatants caused several cultured cell lines, including HeLa and Vero cells, to become slowly distended over a 2- to 4-day period, after which the cells disintegrated [72]. The *C. jejuni cdt* genes have been cloned and sequenced [73]. CDT activity is encoded by three adjacent genes, *cdtA*, *cdtB*, and *cdtC*, which encode proteins predicted to have molecular weights of about 30,000, 29,000, and 21,000, respectively [73, 74]. In the last decade, the gene encoding CDT, *cdtB*, has been found in most *Campylobacter* species [73-78]. Homologues of *cdtB* and CDT activity have also been found in several serotypes of *E. coli* [79-83] and enterohepatic *Helicobacter* species [84, 85]. Other gram-negative bacteria harboring the *cdtB* gene and producing CDT activity include the periodontopathogenic bacterium *Actinobacillus Actinomycetemcomitans* [86-89].

The mechanism of CDT activity is reported to involve the G_2/M cell-cycle phase of certain target eukaryotic cells. At this phase, the CDT toxin causes progressive cytoplasmic and nuclear distension accompanied by increased DNA contents, leading to growth arrest and, ultimately, cell death [78, 82, 90, 91].

SPECIFIC AIMS AND HYPOTHESES

Despite epidemiological, biological and molecular evidence associating the oral infectious agent *Campylobacter rectus* and adverse pregnancy outcomes, certain lingering questions must be addressed. Namely, how is this organism evading the host defense and infecting the fetus and is there a possibility to prevent this from happening.

It is our hypothesis that immunization with heat-killed *C. rectus* 314 will offer protection to fetal growth restriction in the murine model. Specifically, our aims were to study the possibility of protecting the fetus from growth restriction by mean of maternal immunization. Also, we plan to examine the maternal IgG antibody response to *C. rectus* infection in immunized mice versus non-immunized mice.

To investigate the possibility of fetal protection, we utilized an established remote subcutaneous chamber model that has been used to study fetal growth restriction by *C. rectus* [19, 92]. These studies have reported fetal growth restriction induced by maternal *C. rectus* infection and the outline of our experiment mirrored these studies. We compared 3 groups of mice: (1) pregnant mice immunized with heat-killed *C. rectus* and then challenged with live *C. rectus*, (2) pregnant mice not pre-immunized but challenged with live *C. rectus* and (3) a control group not immunized or challenged. Maternal antibody response and fetal growth

restriction were compared between these groups to evaluate possible protection from adverse pregnancy outcomes.

Also, it is our hypothesis that *C. rectus* contains one or more of the virulence genes already identified in other *Campylobacter* species. Specifically, our aim was to investigate the presence of 4 virulence genes in the genome of *C. rectus* via degenerate PCR. The 4 genes we examined were: ***ciaB*** (Invasion protein required for invasion of epithelial cells), ***cdtB*** (Cytolethal distending toxin, known to arrest mammalian cells during the cell cycle), ***cadF*** (Fibronectin binding protein important for cell binding) and ***peb4*** (responsible for adhesion). All of the chosen genes are conserved in *C. jejuni, C. coli, C. lari,* and *C. upsaliensis* while 3 of 4 are conserved in *C. fetus* (*ciaB*, *cdtB* and *cadF*). To investigate their presence in *C. rectus*, we first designed degenerate primers for these genes from the genome of *C. jejuni* 78-14 and *C. coli*. Prior to running the degenerate PCR, we first tested the authenticity of the template DNA and the degenerate primers by means of a positive control PCR. After confirmation that our template DNA was *C. rectus* and our degenerate primers worked, PCR of *C. rectus* with these degenerate primers was done. The PCR products obtained were gel extracted and sequenced.

MATERIALS AND METHODS

Effects of Immunization in the Pregnant Mouse Model

Experimental Groups

Three experimental groups were studied: **Group A** consisted of mice that received immunization prior to challenge with *C. rectus* 314. **Group B** consisted of mice that only received challenge with *C. rectus* 314 while **Group C**, the control group, only received PBS (phosphate buffered saline). There were 5 female mice in test group A, 32 mice in test group B and 27 mice in the control group.

The timeline of the experimental protocol for chamber implantation, immunization with heat-killed *C. rectus*, challenge with *C. rectus* and sacrifice of pregnant mice is summarized in **Figure 2**. Sacrifice was performed on Gd 16.5. At that time, maternal serum samples were collected via cardiac puncture and the fetal weight and length were recorded.

The experiment, data collection and serum samples for Group B and Group C was done and provided by Dr. Yiorgos Bobestsis and Dr. Steven Offenbacher's laboratory.

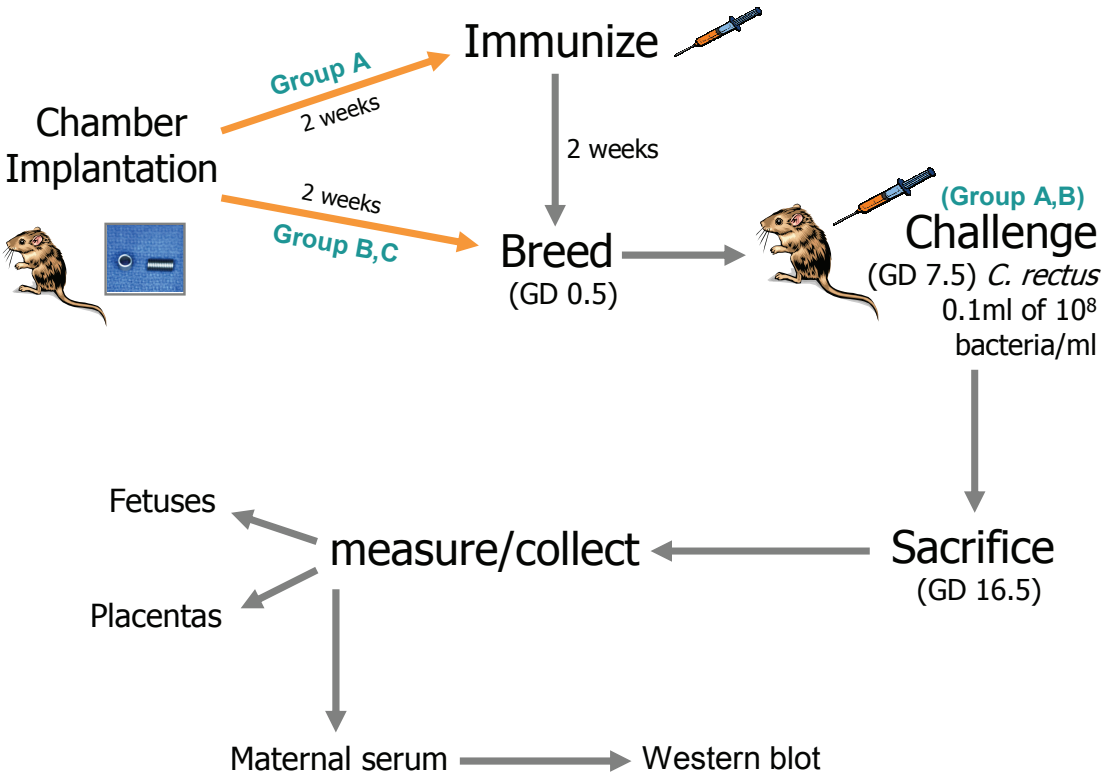

Figure 2. Study design to investigate maternal immune response and fetoprotective effects of immunization.

Animal Husbandry

All procedures were in accordance with the animal welfare guidelines and approved by the University of North Carolina – Chapel Hill Institutional Animal Care and Use Committee. BALB/c mice (Jackson Laboratory, Bar Harbor, MI) were obtained at 4-6 weeks of age, and maintained under controlled, standardized conditions. The mice were housed under controlled and standardized conditions, with 12-hour light-dark cycles (0700-1900 light). Regular mouse diet and water were provided *ad libitum*.

Chamber Implantation

A stainless steel, open-ended cylindrical coil spring (chamber) approximately 1.0 cm x 0.4 cm was inserted subcutaneouly into the female mice. To implant the chamber, each female mouse was anesthetized with 6 mg per kg xylazine (20 mg/ml) and 130 mg per kg ketamine hydrochloride saline solution (100 mg/ml) given in 0.1 ml with normal saline solution injected intra-peritoneally.

Under anesthesia, the neck region of the mouse was shaved and disinfected with 70 % ethanol and betadine. A small incision (~2 cm) was made in the skin of the back with scissors paralleling the line connecting the apical most point of shoulder blades and just coronal to the line. A cylinder shaped dissection was made sub-dermal to the right flank with a pair of needle holders. A sterilized chamber was placed in the dorsal lumbar region, as described by Genco and co workers [93]. The skin was then sutured with 4-0 silk, and the site allowed to heal for at least 2 weeks.

Bacteria Preparation for Immunization and Challenge

C. rectus was cultured on ESTA plates. After 5-8 days, bacteria were collected by gently scrapping the surface of the plate using a sterile cotton swab. The swab was then immersed in sterile PBS (3 ml in a sterile 15 ml conical tube) and rubbed and rotated against the side of the test tube to release the bacteria. The bacterial concentration was evaluated by spectrophotometry, with a measured OD_{600nm}= 1 corresponding to 1 X 10^9 bacteria/ml.

Immunization

The immunization solution was prepared by heating the bacteria in boiling water for 10 minutes. Two weeks after the chamber implantation surgery, 5 mice were immunized by intra-chamber injection of 0.1 ml PBS containing 10^9 bacteria/ml of heat-killed *C. rectus* 314. Mice were then allowed to recover and build immunity for 2 weeks.

Mice Breeding

Sixty-four female mice were mated with male mice of the same strain. The mice were mated overnight. The next morning all female mice were removed from the male cages and examined for vaginal plugs. If plugs were found, that day was recorded as gestation day (Gd) 0.5.

Challenge with *Campylobacter rectus*

On Gd 7.5, pregnant mice in Group C (control group) received an intra-chamber injection of 0.1 ml PBS, while the pregnant mice in test groups A and B received an intra-chamber injection of 0.1 ml PBS containing 10^9 bacteria/ml live *C. rectus* 314 (10^8 CFU).

Monitoring of Animal Responses after Bacterial Challenges

The general response of the animals was monitored during the experimental period. Chamber sloughing, secondary ulceration, cachexia, body weight change before and after *C. rectus* challenge, and death were recorded if any.

Animal Sacrifice and Data Collection

Prior to sacrifice, the female mice were anesthetized with phenobarbital sodium solution (50 mg/ml) given in 0.1 ml increments with normal saline solution injected intra-peritoneally. The abdomen was opened and the uterus dissected. The resorption sites and positions of viable fetuses were recorded. Fetuses were removed from the chorioamniotic sac and their weight and crown-rump length were measured.

Statistical Analysis

In order to account for the fact that the effects of immunization and challenge on littermates are clustered within dams, we used a mixed model method [94]. The statistical comparison of fetal weight between the 3 groups was evaluated with a mixed effects model with a fixed effect for treatment and a random effect for dam, the latter providing adjustment

for intralitter correlation of fetal weight. Similar analyses were applied to fetal length. Data were analyzed using statistical software (SAS Institute, Cary, NC).

Evaluation of Maternal Antibody Response

Sample Collection

Maternal blood was collected by cardiac puncture and stored overnight at 4 °C. The blood clot was removed and serum was then separated by centrifugation at 2000 X g for 20 minutes and then stored at -80 °C until ready for western blot analysis.

Antigen Preparation, SDS-PAGE and Western Blotting

C. rectus cells were harvested for lysate preparation from a 5-8 day old plate of *C. rectus* as described above. The turbidity was adjusted to an OD_{600nm} of 4.0. Cells were then sonicated on ice for 20 minutes. Equal volumes (10 microliters) of the bacterial sample were taken and a 1:3 dilution was made using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA). The samples were centrifuged for 1 min to pellet any cell debris. Supernatants were transferred to another tube and 5 µl of 5 X Laemmli Buffer and 1 µl of β mercaptoethanol were added to each sample. The samples were boiled for 5 min and then applied to 10 % Novex Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Whatman). Blots containing the *C. rectus* protein were blocked with 5 % non-fat milk for 1 h at room temperature. The blots for each sample were then incubated with corresponding mouse sera samples (1:500 dilution) in TBST overnight at 4° C and then incubated with goat anti-mouse IgG horse radish peroxidase polyclonal secondary antibody (1:2000 dilution) in 5% non-fat milk for 1 h at room temperature. Blots were washed with

TBST for 10 min at room temperature, exposed to chemiluminescence reagent (Perkin Elmer Western Lightning), and developed. Equal loading of samples was verified by staining of blots with 0.1 % Ponceau S solution (Sigma) for 5 minutes.

Investigation of Virulence Genes *peb4, ciaB, cadF, cdtB*

The study design utilized to investigate the presence of the above virulence genes in *Campylobacter rectus* is outlined in **Figure 3**.

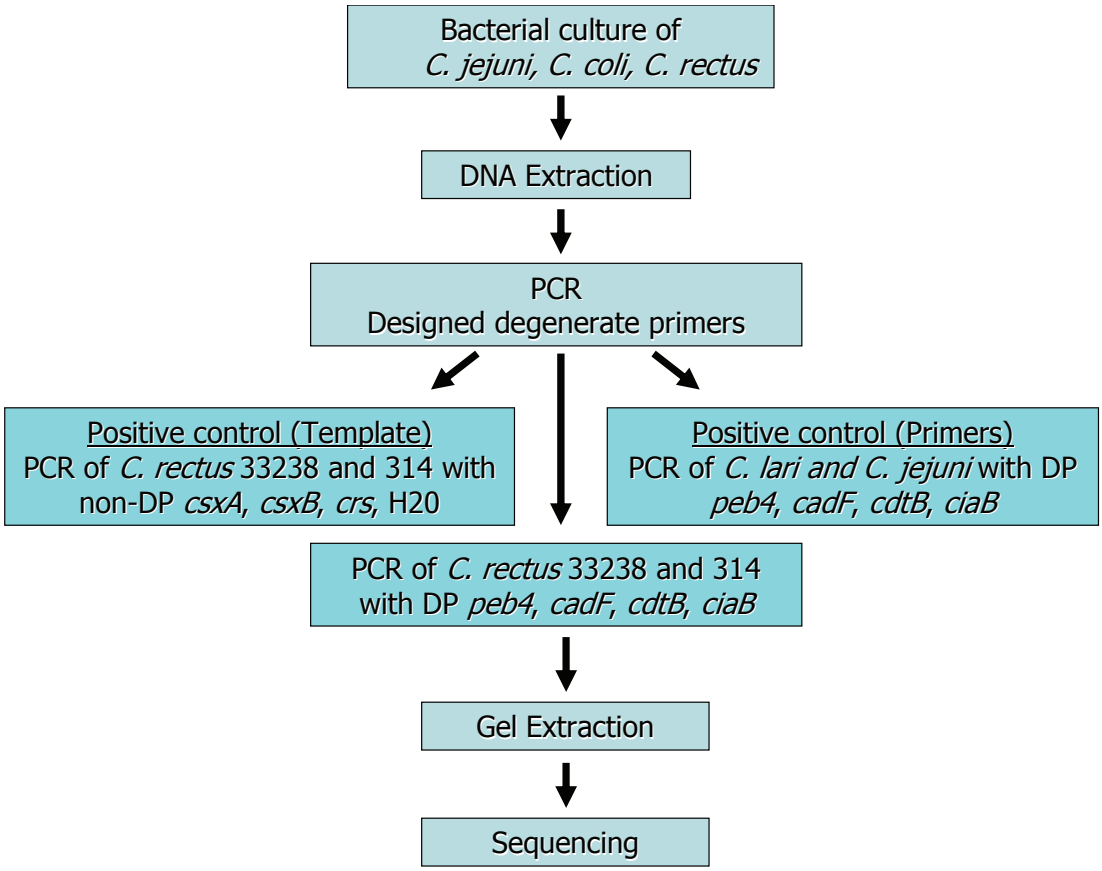

Figure 3. Study design to investigate possible virulence genes in *C. rectus*

Bacterial Strains and Growth

C. rectus ATCC 33238 and 314 were taken from frozen stocks and plated on tryptic soy blood agar plates (Anaerobe Systems) supplemented with formate-fumarate (pH 7.0) to 0.3 %. Cultures were grown for two days in a Thermo Forma Model 1025 anaerobic chamber with anaerobic gas mixture (5 % carbon dioxide, 10 % hydrogen, and 85 % nitrogen). Two plates per strain were harvested with a sterile cotton swab into cold sterile phosphate buffered saline, pH 7.0 (PBS), and centrifuged at 3500 rpm (4 ºC) in a swinging bucket rotor (Beckman, Allegra 25R) for 15 minutes. The supernatant was removed, 1 ml of additional cold PBS was added and pellets were resupended and transferred to prechilled 1.5 ml tubes. The 1.5 ml tubes were centrifuged in a cold microfuge at 13,000 rpm for 1 min. The supernatants were removed, and the pellets frozen immediately at -70 ºC. The pelleted bacteria were stored frozen at -70 ºC until they were used for DNA isolation. Bacterial pellets of these organisms were kindly provided by Dr. Deborah Threadgill, The Department of Genetics at UNC-CH.

DNA Extraction

DNA was extracted as per the manufacturer's instructions using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

PCR

The primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, AI). The design of the specific primers are listed in **Table 1** and the design of degenerate primers are listed in **Table 2**. PCR was performed using the HotStart Taq DNA polymerase Kit (Qiagen,

Valencia, CA) following the manufacturer's instructions. Specifically: 5 µl of 10X PCR buffer solution, 2 µl of 10 mM dNTPs, 2.5 µl of 25 mM $MgCl^2$, 1.5 µl of each primer (50 µM), 2 µl of template DNA (100 ng), and 2.5 U of Taq polymerase were mixed and the final reaction volume was brought to 50 µl by adding double-distilled water. A DNA thermal cycler (GeneAmp) was used for amplification using the cycling conditions listed in **Table 3**. A 15 µl sample of the amplified material was subjected to electrophoresis at 80 mV on 2 % TAE agarose gel. A 100 bp DNA ladder (Promega Corporation, Madison WI, USA) was used as a size marker. The gel was stained with ethidium bromide (Eugene, Oregon, USA) and a photograph was taken under UV light.

Table 1. Specific primer sequences

Gene	Primer Sequences (5′~ 3′)	Annealing Temperature (°C)	Approximate predicted sizes (bp)
csxA		55	2000
F	TGTCCCTAACCCAGTCCCAT		
R	ATCGTATCGTCTCCGTCGTTA		
csxB		55	2000
F	ATGTCCCTAACCCAGTCCCAT		
R	GATTATTGCCGCTTCCCAGAT		
crs		52	2000
F	ATGGCTTTAACACAGACACA		
R	CTCTTACAGTTAGTCTTAGAT		

Table 2. Sequences of degenerate primers

Gene	Degenerate primer sequences (5′~ 3′)	Annealing Temperature (°C)	Approximate predicted sizes (bp)
cdtB		45	500
F	HACTTGGAATTTRCAAGG		
R	ATCBCCBRHWATCATCC		
cadF		45	550
F	AWTWGAAMTHACTCCWAC		
R	GCACCYTYTCTTGG		
ciaB		45	570
F	CCWTTRCAARTRGGWCATC		
R	ARVTCHTCTTSYTCATG		
peb4		45	350
F	GAYACWGAAGTRARTGGA		
R	AATRATATYYTTAGCTTCTT		

IUB codes for mixing bases

B= C,G OR T
D= A,G OR T
H= A,C OR T
V= A,C OR G
R= A OR G
Y= C OR T
K= G OR T
M= A OR C
S= G OR C
W= A OR T

Table 3. PCR cycling conditions

Steps	*csx* and *crs* primers	Degenerate primers
Initial denaturation	95°C for 5 min	95°C for 5 min
Denaturation	95°C for 30s	95°C for 30 sec
Annealing	55°C for 30s (52 for 30s for *crs*)	45°C 5 for 30 sec
Extension	72°C for 1 min	72°C for 1 min
Number of cycles	thirty times	thirty times
Extension	72°C for 7 min	72°C for 7 min
	end	end

Gel Extraction

DNA was purified from agarose gels using the Qia quick® gel extration kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. Five PCR products were extracted and submitted for sequencing.

Sequencing

Nucleotide sequencing was carried out by the UNC-CH sequencing core. Sequences obtained were analyzed using the BLAST program to determine whether any homology existed to any known *Campylobacter* spp. sequences or any other organism. (http://www.ncbi.nlm.nih.gov/BLAST/)

RESULTS

Effects of Immunization in the Pregnant Mouse Model

Data of Experimental Groups at Gd 16.5

Fetuses were measured and maternal serum was collected for experimental groups A, B and C. The sample size of Group B and C was considerably larger than that of Group A regarding the number of dams and number of fetuses studied. However, the unequal group sizes did not hinder evaluation of statistical significance between the groups when comparing fetal weight and fetal length.

Group A (mice that received immunization prior to bacterial challenge) consisted of 5 dams that had a total of 28 fetuses. The mean fetal weight of this group was 0.49 gms (SE 0.017) and the mean fetal length was 1.46 cm (SE 0.028).

Group B (mice that did not receive immunization prior to bacterial challenge) consisted of 32 dams that had a total of 193 fetuses. The mean fetal weight was 0.43 gms (SE 0.007). Regarding the evaluation of fetal length, data for only 27 of these dams were available and provided for analysis. These 27 dams had a total of 161 fetuses and the mean fetal length was 1.34 cm (SE 0.012).

Group C/Control (mice that did not receive either immunization or bacterial challenge) consisted of 27 dams that had a total of 144 fetuses. The mean fetal weight of this group was 0.48 gms (SE 0.008) and the mean fetal length was 1.45 cm (SE 0.012).

Comparison of Fetal Weight and Fetal Length at Gd 16.5

When comparing the mean fetal weights and length, there was a significant difference between group A and B and between group B and C ($p<0.001$). However, there was no significant difference between group A and C. The recorded fetal weight and length measurements at the time of sacrifice Gd 16.5 are summarized in **Table 4** and graphically depicted in **Figure 4 and 5**.

		GROUP A Immunized and Challenged	GROUP B Not Immunized and Challenged	GROUP C Not Immunized and Not Challenged (Control)
Fetal Weight (gram)	Mean (SE)	0.49 (0.017)*	0.43 (0.007)**	0.48 (0.008)
	Number of Fetuses	28	193	144
	Number of Dams	5	32	27
Fetal Length (cm)	Mean (SE)	1.46 (0.028)*	1.34 (0.012)**	1.45 (0.012)
	Number of Fetuses	28	161	144
	Number of Dams	5	27	27

p-value < 0.001 using the Mixed model

* denotes statistical significance between Group A and B but not C
** denotes statistical significance between Group B and C, B and A but not between A and C

Table 4. Summary of maternal and fetal data collected.

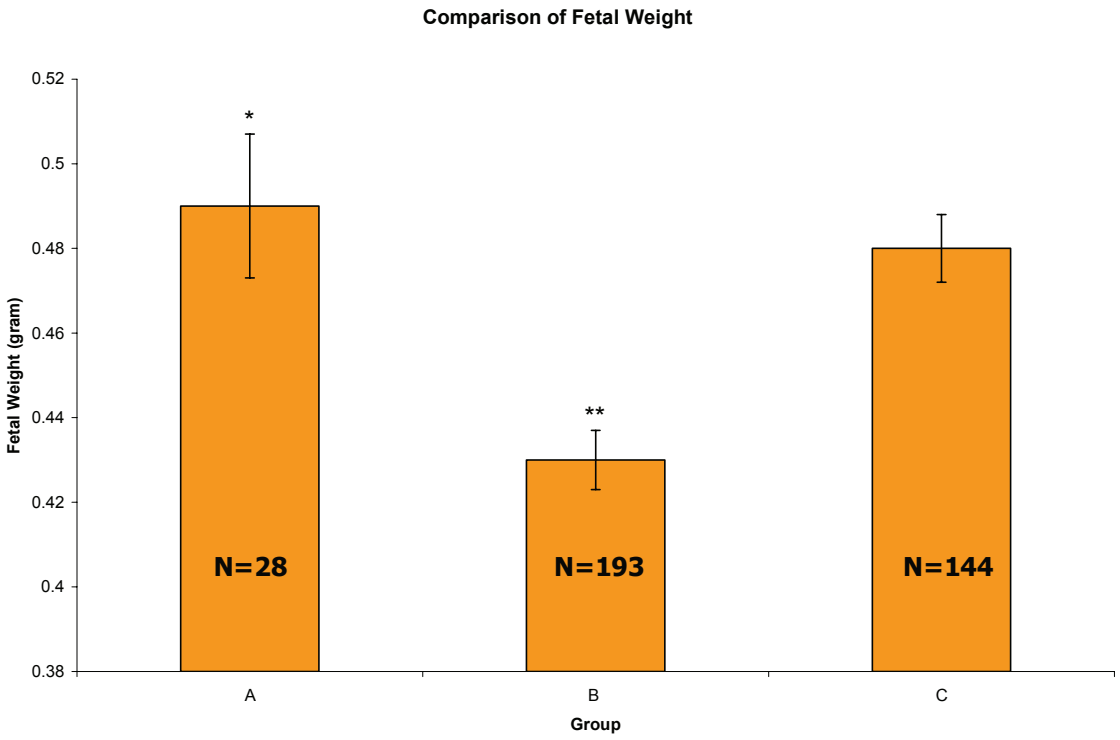

Figure 4. Comparison of fetal weight at Gd 16.5. Error bars represent the standard error of the mean.

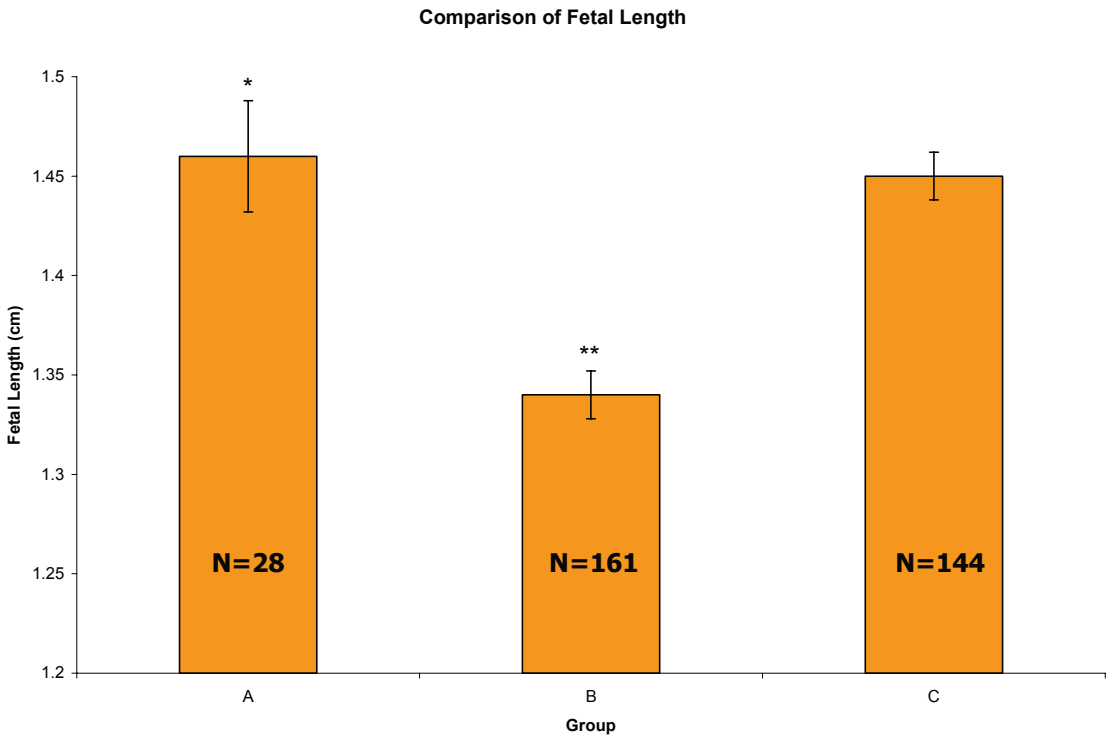

Figure 5. Comparison of fetal length at Gd 16.5. Error bars represent the standard error of the mean.

Evaluation of Maternal Antibody Response

Western Blot

The presence and level of maternal antibody response was evaluated by the western blot technique and shown in **Figure 6.** The results show an overall marked elevation of IgG antibody to *Campylobacter rectus* 314 proteins in maternal serum of Group A compared to Groups B and C. Specifically, Group A displayed numerous bands on the blot, depicting antibody production for proteins of many different sizes. These bands display vigorous IgG antibody binding for both large and small proteins.

Although the maternal serum in Group B had a decreased total amount of IgG response compared to Group A, the blot depicts a more intense response at two sites. These sites represent IgG binding to protein sizes of approximately 140 kDa and 50 kDa. Additional weak bands are noted on the blot, however, they are very light (signifying minimal antibody response) and comparable to the light bands found in the control group.

Equal loading of total protein along the lanes was confirmed by staining the blot with 0.1 % Ponceau S solution as shown in **Figure 7**.

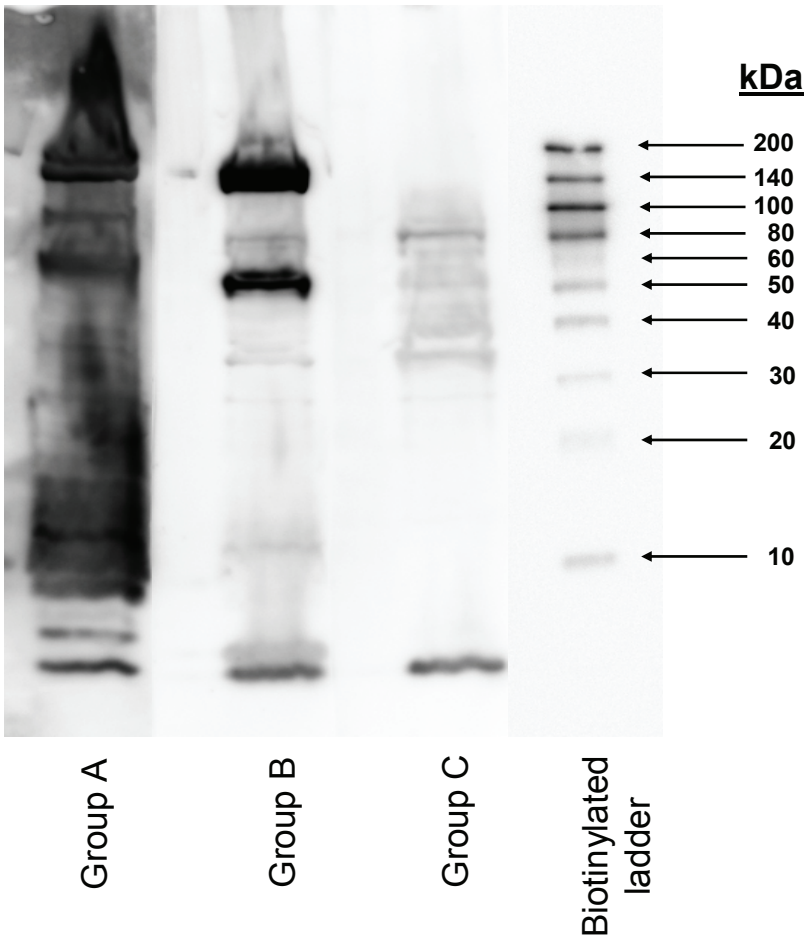

Figure 6. Western blot

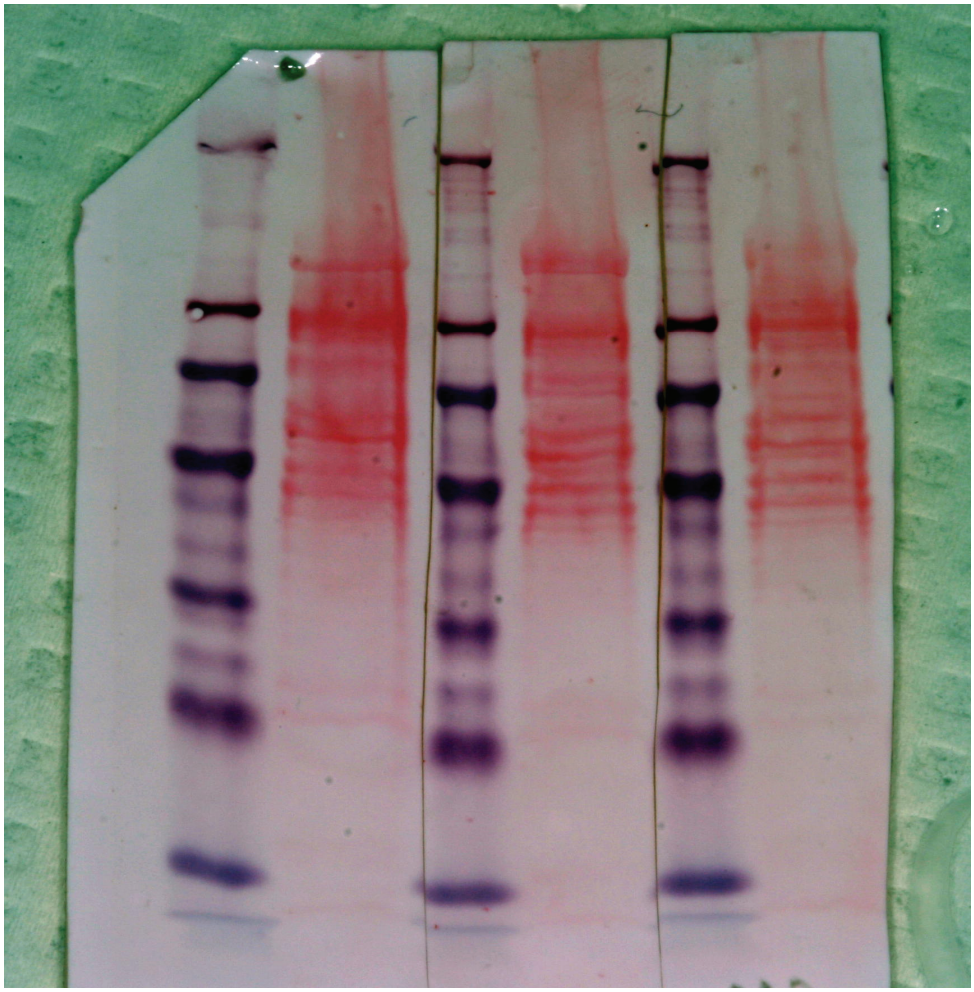

Figure 7. Loading control - stain with 0.1 % Ponceau S solution (Sigma).

Investigation of Virulence Genes *peb4*, *ciaB*, *cadF*, *cdtB*

PCR of Positive Control (Template)

To confirm that the target bacterial DNA belonged to *C. rectus*, we carried out PCR for *C. rectus* 33238 and *C. rectus* 314 using specific primers for known virulence genes (*csxA*, *csxB* and *crs*). PCR results for *C. rectus* 314 are displayed in **Figure 8**.

Lane 1 of the 1% agarose TAE gel contains a 1 kb ladder. Lane 2 contains a PCR product obtained from *C. rectus* DNA using specific primers for the *csxA* gene. The PCR product amplified was the expected appropriate size of 2000 bp. Lane 3 contains a PCR product obtained from *C. rectus* DNA using specific primers for the *csxB* gene. Again, the expected appropriate size of 2000 bp was amplified. Interestingly, Lane 3 contains substantially more product than lane 2 or lane 4. Lane 4 contains a PCR product obtained from *C. rectus* DNA using specific primers for the *crs* gene. This band also displayed amplification of 2000 bp. Lane 4 displays a very light band, signifying a reduced quantity of product, possibly due to the annealing temperature utilized. Lane 5 contains a "no-template" control. Here, distilled water was used instead of DNA template to demonstrate that the PCR products obtained were not a result of contamination. As expected, bands are not present in this lane.

Due to the specific amplification of PCR products of appropriate sizes, it was deduced that the template DNA was *C. rectus* 314. Similar results were obtained from PCR of *C. rectus* 33238 with these specific primers (not shown).

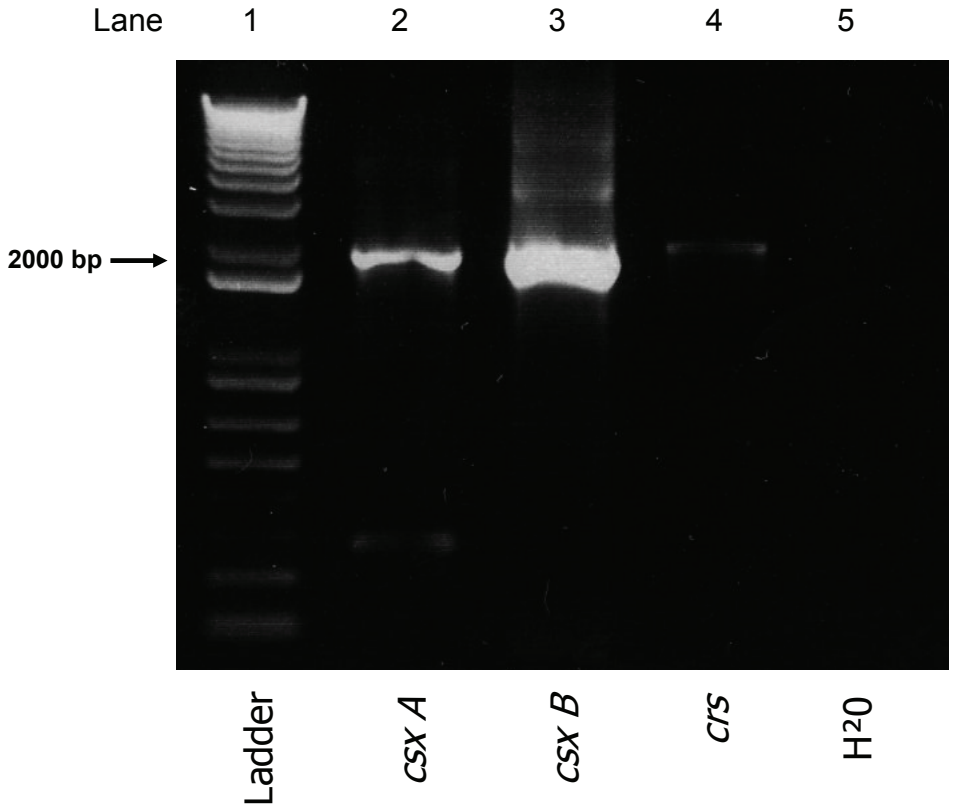

Figure 8. PCR products from *C.rectus* 314 obtained using specific primers for *csxA*, *csxB* and *crs* genes. The results show bands of the expected sizes of approximately 2000 bp.

PCR of Positive Control (Primers)

To confirm that the newly designed degenerate primers were effective, PCR was carried out on template DNA from organisms from which the degenerate primers were designed (*C. jejuni, C. lari, C. coli* and *C. upsaliensis*). The result of this PCR is displayed in **Figure 9**.

Lane 1 of the 1% agarose TAE gel contains a 1 kb ladder. Lane 2 contains PCR products from *C. jejuni* 78-14 obtained using degenerate primers for the gene *peb4*. As expected, the products were 350 bp. Lane 3 contains PCR products from *C. jejuni* 78-14 obtained using degenerate primers for gene *cadF* having the expected appropriate size of 550 bp. Lane 4 contains PCR products from *C. jejuni* 78-14 obtained using degenerate primers for gene *cdtB* having the expected appropriate size of 500 bp. Lane 5 contains PCR products from *C. jejuni* 78-14 obtained using degenerate primers for gene *ciaB* having the expected appropriate size of 570 bp.

Lane 6 contains three distinct bands of PCR products from *C. lari* obtained using degenerate primers for gene *peb4*. Although multiple bands were found, the expected appropriate band displaying product size of 350 bp was present. The two other bands found were 200 bp and 500 bp. Lane 7 contains PCR products from *C. lari* obtained using degenerate primers for gene *cadF* having the expected appropriate size of 550 bp. Lane 8 contains PCR products from *C. lari* obtained using degenerate primers for gene *cdtB* having the expected appropriate size of 500 bp. Lane 9 contains PCR products from *C. lari* obtained using degenerate primers for gene *ciaB* having the expected appropriate size of 570 bp. The results of this PCR show bands of the expected sizes for each gene in question. This confirms

that the degenerate primers are effective in amplifying the appropriate sequences from the organisms they were designed from.

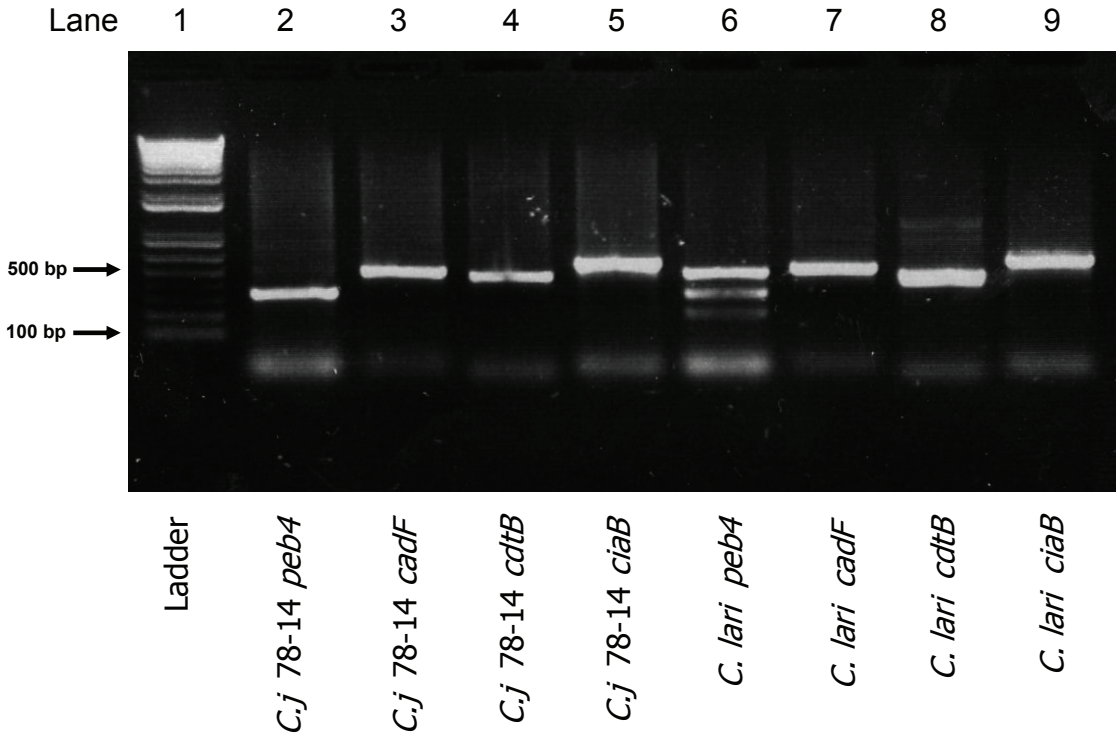

Figure 9. PCR products of *C. jejuni* 78-14 using degenerate primers for *peb4, cadF*, *cdtB* and *ciaB* constructed from the *C. jejuni* 78-14 genome are displayed in Lanes 2-5. PCR products of *C. lari* using degenerate primers for *peb4, cadF*, *cdtB* and *ciaB* constructed from the *C. lari* genome are displayed in Lanes 6-9.

PCR of *C. rectus* 314 With Degenerate Primers

After confirmation that the template DNA was from *C. rectus* and that the degenerate primers were effective in amplifying our selected virulence genes in *C. jejuni* and *C. lari*, PCR of *C. rectus* with these degenerate primers was done and the results are displayed in **Figure 10**.

Lanes 1 and 10 of the 1% agarose TAE gel contains a 1 kb ladder. Lanes 2, 4, 6 and 8 contain PCR products from *C. jejuni* 78-14 obtained using degenerate primers for genes *peb4, cadF*, *ciaB* and *cdtB* respectively. These results have already been established from a previous PCR depicted in **Figure 9**. and they serve as the control in this PCR. Briefly, the sizes of products for lanes 2, 4, 6 and 8 are 350 bp, 550 bp, 570 bp and 500 bp respectively.

Lane 3 contains PCR products of *C. rectus* 314 obtained using degenerate primers for *peb4*. Two faint bands (low quantity of product) were obtained of approximately 800 bp and 570 bp. Neither band was close to the appropriate expected size of 350 bp.

Lane 5 contains PCR products of *C. rectus* 314 with degenerate primers for *cadF*. Two light bands were obtained, one of which appears to be of the appropriate expected size of 550 bp. The other was approximately 350 bp.

Lane 7 contains PCR products of *C. rectus* 314 obtained using degenerate primers for *ciaB*. Two bands were obtained, one light and one intermediate in quantity. Neither band was of the appropriate expected size of 570 bp, however, the lighter band was close to this size (approximately 500 bp) while the intermediate band was approximately 1000 bp.

Lane 9 contains PCR products of *C. rectus* 314 obtained using degenerate primers for *cdtB*. Two bands were obtained, one light and one strong (high in quantity). Neither band was of the appropriate expected size of 500 bp, however, both bands were very close to this

size. The lighter band appears to be approximately 600 bp and the stronger band appears to be approximately 400 bp.

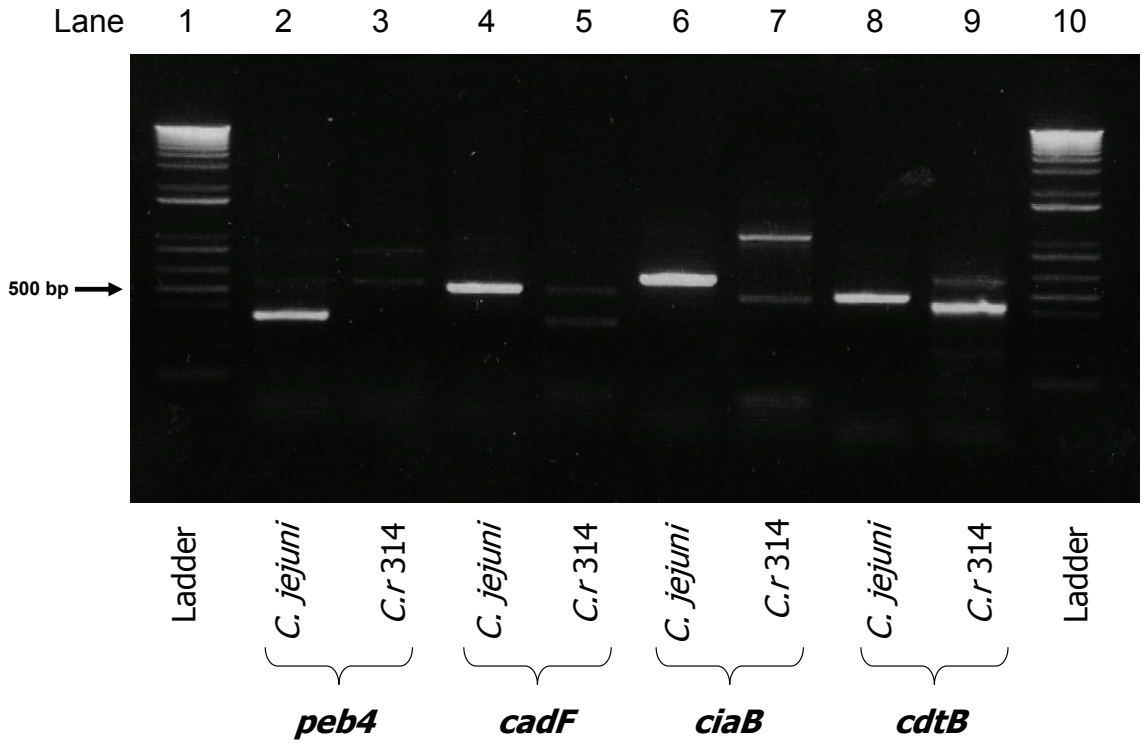

Figure 10. PCR products of *C. rectus* 314 and *C. jejuni* (control) using degenerate primers for genes *peb4*, *cadF*, *ciaB* and *cdtB*.

Sequencing and Sequence Analysis

Five PCR products were gel extracted and sent for sequencing at UNC-CH sequencing core. These included one *ciaB* (500 bp) from *C. rectus* 314, one *cadF* (550 bp) from *C. rectus* 314, one *cdtB* (400 bp) from *C. rectus* 314 and two *cdtB* (500 bp and 550 bp) from *C. rectus* 33238. Four of the five PCR products submitted for sequencing yielded workable results. After BLAST analysis, the stretches of homology obtained were low on a nucleotide level. Therefore, the nucleotide sequences were translated to protein using the ExPASy program (www.expasy.org) and BLAST analysis was carried out. BLAST searches of protein sequences found the following matches:

- For *cadF* in *C. rectus* 314, there was 53% identity to cytochrome c552 of *C. jejuni* RM1221 based on a translated protein BLAST against the protein database.
- For *cdtB* in *C. rectus* 314, there was a 75% identity to NADP-dependent malic enzyme from. *C. fetus* subsp. *fetus*.
- For *cdtB* in *C. rectus* 33238, there was a 70% identity to NADP-dependent malic enzyme from *C. fetus* subsp. *fetus*.
- For *cdtB* in *C. rectus* 33238, there was a 67% identity to mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase from *C. fetus* subsp. *fetus*.

DISCUSSION

Regarding the investigation of *C. rectus* virulence, the present study was designed to test the hypothesis that certain genes responsible for virulence that are conserved in some *Campylobacter* species are also conserved in *C. rectus*. To investigate the presence of conserved genes, we utilized degenerate primers. A PCR primer sequence is called *degenerate* if some of its positions have several possible bases [95]. Therefore, degenerate primers are particularly useful in amplifying homologous genes from different organisms [96-98]. Homologous genes display regions where they are highly conserved and also regions where they have evolved and are divergent. Degenerate primers are designed within the conserved regions of the target gene, accounting for random variations that have occurred over time. Due to their non-specificity, degenerate primers can be used to isolate orthologous genes encoding proteins that belong to known protein families [97].

To that end, degenerate primers were formulated for previously characterized virulence genes in *C. jejuni, C. lari, C. coli and C. upsaliensis*. In our investigation of these genes in *C. rectus*, we obtained 5 PCR products that were suitable for sequencing. Four of these matched known genes in the BLAST database. DNA degradation, insufficient DNA or sequencing errors could account for the failure of one of these samples to be read.

The 4 PCR products that could be read were believed to be *cadF* or *cdtB* (*cad C.r* 314, *cdtB C.r* 314, 2 x *cdtB C.r* 33238). Two PCR products were investigated for *cdtB C.r* 33238 as it was not known which band potentially contained the sequences for *cdtB*.

BLAST analysis of our nucleotide sequences failed to find any strong matches for known genes in the database. One possible reason may be that the target DNA sequence from *C. rectus* may be very far removed from other *Campylobacter* species. They may share the same gene but may not be similar at the nucleotide level.

The reason amino-acid sequences were analyzed after DNA analysis failed was that different combination of bases can code for the same amino acid. Therefore, we may see a lot of diversity at the nucleotide level but that may not hold true at the protein level. Generally, it is found that amino acid sequences are more conserved than nucleotide sequences. To that end, if a match is not found at the nucleotide level, it is worthwhile to investigate matches at the protein level as there may be decreased diversity. We had a PCR product the same size as the size of *cadF* in *C.r* 314 and a protein BLAST revealed an identity with cytochrome c552 of *C. jejuni* RM1221. Although we did not get a match for *cadF* as we expected, we did get a protein match with *Campylobacter jejuni*. Therefore, although we did not amplify the *cadF* gene from *C. rectus*, we did amplify a sequence belonging to *C. rectus* as these protein sequences match with those of other *Campylobacter* species. As there is minimal sequence data relating to the *C. rectus* genome, any sequences obtained from this organism are valuable. For instance, the cytochrome c552 of *C. jejuni* RM1221 has not been previously identified in *C. rectus* 314. Therefore, this is the first account of *C. rectus* 314 sequences that share similarity to cytochrome c552 of *C. jejuni* RM1221. Similarly, this is the first account of *C. rectus* 314 sequences that share similarity to NADP-dependent malic enzyme and

mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase from *C. fetus* subsp. *fetus*.

Previous murine model studies utilizing sensitized mice have reported significant FGR and adverse pregnancy outcomes upon remote subcutaneous maternal *C. rectus* infection [19, 92]. The same model was used to determine the role of immunization to this organism, specifically, if immunization protected the fetus from these adverse events. In addition to fetal measurements, maternal antibody response was evaluated via investigation of IgG in maternal serum.

Overall, immunization had a significant effect on the mean fetal weight and length. At the day of sacrifice Gd 16.5, the mean fetal weight and length of immunized mice were significantly higher than non-immunized mice. However, these measurements were not significantly different from the control mice. The data collected from this experiment strongly suggests immunization protects against FGR, as immunized mice do not display FGR when challenged with *C. rectus* 314 and their fetuses have the same weight and length as mice given PBS.

Further evidence of protection is seen in the IgG response of mice in the experimental groups. As depicted by western blotting, immunized mice displayed a robust IgG response to many sizes of *C. rectus* proteins compared to non-immunized and the control mice. The reason Group-A mice display numerous bands on the blot is likely due to receiving heat-killed *C. rectus* 314. Boiling and sonication effectively lysed the organism and therefore, its inner and outer proteins were exposed. This facilitated recognition and subsequent antibody production of multiple *C. rectus* proteins of many different sizes. In comparison, Group-B mice were only exposed to intact *C. rectus* 314 and therefore recognized and produced

antibodies of substantially fewer proteins of the outer membrane. Specifically, the western blot displays strong bands for mainly two *C. rectus* proteins of size 150 kDa and 55 kDa. This result is suggestive of antibody recognition of the *C. rectus* S-layer surface protein, described as having a molecular mass of 150-166 kDa.

The specific size of proteins that may play a large role in virulence of *C. rectus* is best explained by assessing the differences between Group A and Group B in production of IgG for these various protein sizes. Therefore, the proteins recognized by Group A but not recognized by Group B are the most likely candidates to cause FGR. Specifically, IgG production against these proteins offers protection against FGR in Group A mice, while lack of IgG response to these proteins in Group B mice may explain the FGR in this group. Coincidentally, the 4 virulence genes we investigated in our experiment code for proteins of sizes 31 kDa (*peb4*), 37 kDa (*cadF*), 29 kDa (*cdtB*) and 73 kDa (*ciaB*). Of these, it appears Group B mice only displayed a band for size 37 kDa (suggesting it may be *cadF*) while Group A mice displayed bands for all of these protein sizes. Although we have not confirmed the exact sequences of these proteins, their sizes are highly suggestive of the proteins produced by the genes we investigated.

CONCLUSION

- Overall, the mean fetal weight and fetal length of immunized mice were significantly higher than those of non-immunized mice.
- The serum of immunized mice displayed an overall marked elevation of IgG antibody to *C. rectus* 314 proteins compared to non-immunized mice.
- Our data suggest that immunization with heat-killed *C. rectus* 314 offers protection from fetal growth restriction in the murine model
- Although our degenerate PCR products did not amplify known *Campylobacter* virulence genes, the results leading up to the last few steps of the experiment were highly encouraging and validate further study.

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